SUPPORTING INFORMATION

An Orally Bioavailable Chemical Probe of the Lysine Methyltransferases EZH2 and EZH1

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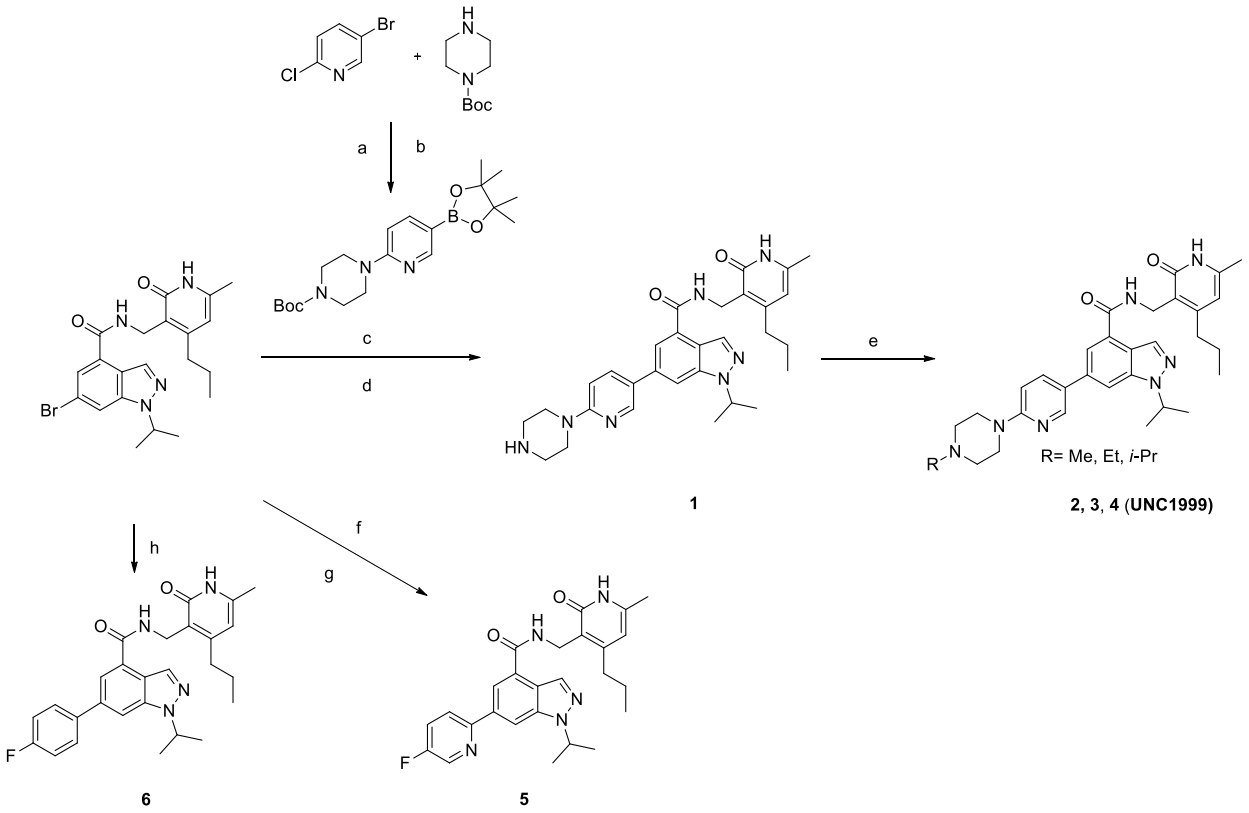
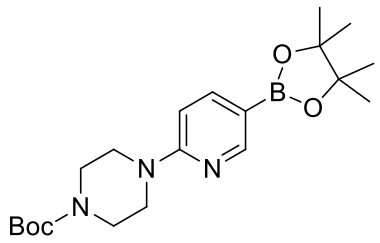
Chemistry General Procedures. HPLC spectra for all compounds were acquired using an Agilent 6110 Series system with UV detector set to 254 nm. Samples were injected (5 μL) onto an Agilent Eclipse Plus 4.6 x 50 mm, 1.8 μM, C18 column at room temperature. Method 1: A linear gradient from 10% to 100% B (MeOH + 0.1% acetic acid) in 5.0 min was followed by pumping 100% B for another 2 minutes with A being H_2O + 0.1% acetic acid. Method 2: A linear gradient from 50% to 100% B (MeOH + 0.1% acetic acid) in 5.0 min was followed by pumping 100% B for another 2 minutes with A being H_2O + 0.1% acetic acid. The flow rate was 1.0 mL/min. Mass spectra (MS) data were acquired in positive ion mode using an Agilent 6110 single quadrupole mass spectrometer with an electrospray ionization (ESI) source. Nuclear Magnetic Resonance (NMR) spectra were recorded at Varian Mercury spectrometer with 400 MHz for proton (^{1}H NMR) and 100 MHz for carbon (^{13}C NMR); chemical shifts are reported in ppm (δ). Preparative HPLC was performed on Agilent Prep 1200 series with UV detector set to 254 nm. Samples were injected onto a Phenomenex Luna 75 x 30 mm, 5 μM, C_{18} column at room temperature. The flow rate was 30 mL/min. A linear gradient was used with 10% (or 50%) of MeOH (A) in 0.1 % TFA in H_2O (B) to 100% of MeOH (A). HPLC was used to establish the purity of target compounds, all compounds had > 95% purity using the HPLC methods described above unless noted otherwise. High-resolution (positive ion) mass spectrum (HRMS) for compound UNC1999 was acquired using a Thermo LTqFT mass spectrometer under FT control at 100000 resolution.

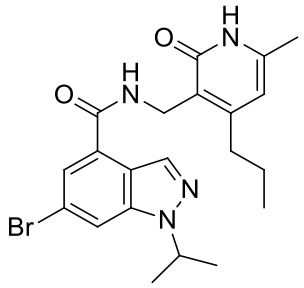
Scheme S1. Synthesis of the compounds in Table 1.



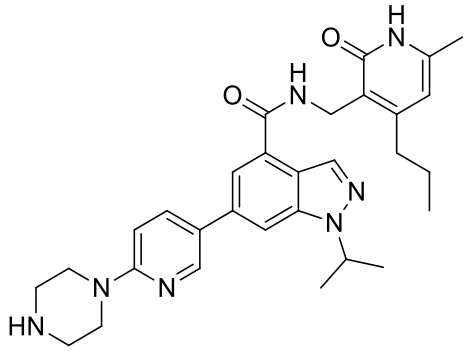
Reagents and conditions: (a) K_2CO_3, NMP (1-methyl-2-pyrrolidinone), 120°C, 50%; (b) 4,4,4',4',5,5,5',5'-octamethyl-2,2'-bi(1,3,2-dioxaborolane), KOAc, 1,4-dioxane, $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, 80°C, 55%; (c) KOAc, 1,4-dioxane, H_2O, $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, reflux, 78%; (d) HCl (aq), rt, 95%; (e) formaldehyde solution (37 wt.% in H_2O) / acetaldehyde / acetone, $NaBH_3CN$, HOAc, MeOH, 0°C - rt, 48-86%; (f) bis(pinacolato)diboron, KOAc, $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, 1,4-dioxane, 80°C, Ar; (g) 2-bromo-5-fluoropyridine, $Pd(PPh_3)_4$, K_2CO_3, 1,4-dioxane, H_2O, 90°C, 61% (two steps); (h) K_2CO_3, (4-fluorophenyl)boronic acid, $Pd(PPh_3)_4$, 1,4-dioxane, H_2O, microwave, 150°C, 73%.



***tert*-Butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate**. 5-Bromo-2-chloropyridine (10 g, 52 mmol), *tert*-butyl piperazine-1-carboxylate (29 g, 156 mmol), and potassium carbonate (22 g, 156 mmol) were mixed in NMP (50 mL) and heated to 120°C overnight. The mixture was then cooled to room temperature and diluted with water. The solid was collected by filtration, washed with water and dried under vacuum to give *tert*-butyl 4-(5-bromopyridin-2-yl)piperazine-1-carboxylate as a white solid (9 g, 50% yield). *tert*-Butyl 4-(5-bromopyridin-2-yl)piperazine-1-carboxylate (5 g, 14.6 mmol), 4,4,4',4',5,5,5',5'-octamethyl-2,2'-bi(1,3,2-dioxaborolane) (4.46 g, 17.6 mmol), and potassium acetate (2.15 g, 22 mmol) were mixed with 1,4-dioxane (120 mL) in a flask. To this mixture, 1,1'-bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex (600 mg, 0.73 mmol) was added under argon atmosphere at room temperature. The mixture was heated to 80°C for 5 h and cooled to room temperature. The resulting mixture was then diluted with EtOAc and washed with water. The combined organic layers were dried over sodium sulfate. After concentrated *in vacuo*, the crude product was purified by silica chromatography (PE/EA = 5/1) to afford the title compound as a white solid (3.2 g, 55% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 – 8.51 (m, 1H), 7.83 (dd, J = 8.6, 1.9 Hz, 1H), 6.59 (d, J = 8.5 Hz, 1H), 3.60 (dd, J = 6.6, 3.2 Hz, 4H), 3.52 (dd, J = 6.7, 3.2 Hz, 4H), 1.48 (s, 9H), 1.31 (s, 12H).

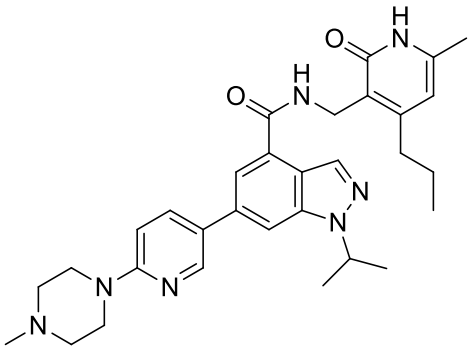


6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide. This compound was synthesized according to the procedures reported previously.[1]

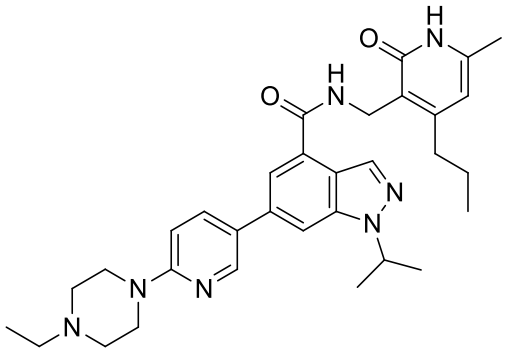


1-Isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-(6-(piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (1). 6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (1 g, 2.3 mmol), *tert*-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2- dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate (965 mg, 2.48 mmol), potassium acetate (680 mg, 6.9 mmol) were mixed with 1,4-dioxane (20 mL) and water (5 mL). To the mixture was added 1,1'-bis(diphenylphosphino) ferrocene-palladium(II)dichloride dichloromethane complex (190 mg, 0.23 mmol) under argon atmosphere at room temperature. The resulting mixture was heated to reflux for 5 h, then cooled and diluted with EtOAc. The mixture was washed with water, dried over sodium sulfate and concentrated *in vacuo*. The crude product was purified by silica chromatography (DCM/MeOH = 40/1) to give

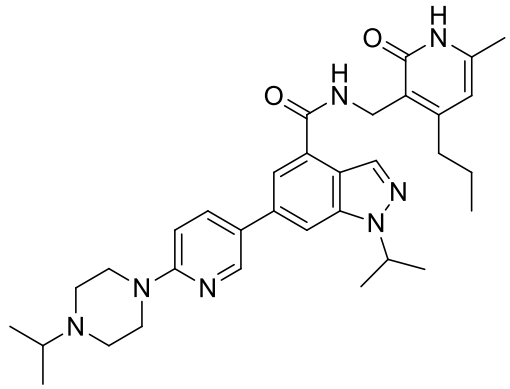
tert-butyl 4-(5-(1-isopropyl-4-(((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)carbamoyl)-1H-indazol-6-yl)pyridin-2-yl)piperazine-1-carboxylate as a white solid (1.1 g, 78% yield). This product was then dissolved in a saturated solution of hydrogen chloride in ethyl acetate. The mixture was stirred at room temperature overnight. The solvent was removed *in vacuo* to give the title compound **1** as a yellow solid (1 g, 95% yield). ^{1}H NMR (400 MHz, d_4-MeOH) δ 8.49 (d, J = 2.3 Hz, 1H), 8.35 (s, 1H), 7.93 (dd, J = 8.9, 2.6 Hz, 1H), 7.88 (s, 1H), 7.75 (d, J = 1.2 Hz, 1H), 6.84 (d, J = 8.9 Hz, 1H), 6.10 (d, J = 0.6 Hz, 1H), 5.06 (dt, J = 13.3, 6.6 Hz, 1H), 4.58 (s, 2H), 3.52 (dd, J = 6.0, 4.3 Hz, 4H), 2.91 (dd, J = 5.9, 4.3 Hz, 4H), 2.76 – 2.67 (m, 2H), 2.24 (s, 3H), 1.62 (td, J = 15.0, 7.5 Hz, 2H), 1.56 (t, J = 5.7 Hz, 6H), 1.00 (t, J = 7.3 Hz, 3H). HPLC (method 1): 95%; t_R 4.33 min; MS (ESI): 528 $[M+H]^+$.



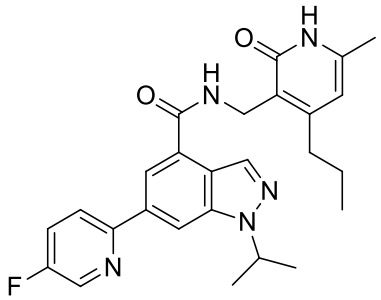
1-Isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-(6-(4-methylpiperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (2). Compound **2** was synthesized according to the procedures reported previously.[1]



6-(6-(4-Ethylpiperazin-1-yl)pyridin-3-yl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (3). Compound **1** (50 mg, 0.089 mmol), acetaldehyde (50 μL, 0.89 mmol) and acetic acid (~0.5 μL, 0.009 mmol) were mixed with methanol (1 mL). To the mixture was added sodium cyanoborohydride (17 mg, 0.267 mmol) at 0°C. After being stirred overnight at room temperature, the mixture was purified by preparative HPLC (10% – 100% methanol / 0.1% TFA in H_2O) to afford compound **3** as a white solid (24 mg, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.66 (s, 1H), 8.48 (d, J = 2.5 Hz, 1H), 8.39 (s, 1H), 8.00 (t, J = 5.7 Hz, 1H), 7.76 (dd, J = 8.8, 2.6 Hz, 1H), 7.71 (d, J = 1.2 Hz, 1H), 7.58 (s, 1H), 6.67 (d, J = 8.8 Hz, 1H), 5.93 (s, 1H), 4.87 (dp, J = 13.1, 6.6 Hz, 1H), 4.67 (d, J = 5.8 Hz, 2H), 3.75 – 3.58 (m, 4H), 2.75 – 2.67 (m, 2H), 2.65 (s, 4H), 2.56 (dd, J = 14.2, 7.1 Hz, 2H), 2.16 (s, 3H), 1.65 (dd, J = 15.2, 7.5 Hz, 2H), 1.58 (d, J = 6.7 Hz, 6H), 1.18 (t, J = 7.2 Hz, 3H), 1.01 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.43, 165.87, 158.74, 154.75, 146.68, 143.12, 139.93, 136.66, 136.29, 132.38, 129.03, 126.05, 121.73, 120.60, 120.07, 109.12, 108.65, 106.92, 52.55, 52.42, 50.53, 44.90, 36.26, 35.35, 23.79, 22.27 (two carbons), 18.90, 14.09 (two carbons), 11.64, 1.15. HPLC (method 1): 95%; t_R 4.46 min; MS (ESI): 556 $[M + H]^+$.

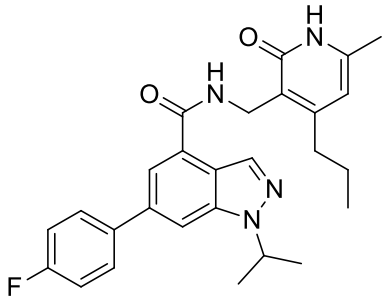


1-Isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (UNC1999 (4)). The procedures used for preparation of compound **3** were followed for synthesis of the title compound. This compound was obtained as a white solid (21 mg, 78%). ^{1}H NMR (400 MHz, d_4-MeOH) δ 8.48 (d, J = 2.4 Hz, 1H), 8.36 (s, 1H), 7.91 (dd, J = 8.9, 2.6 Hz, 1H), 7.87 (s, 1H), 7.75 (d, J = 1.2 Hz, 1H), 6.81 (d, J = 8.9 Hz, 1H), 6.09 (s, 1H), 5.06 (dt, J = 13.3, 6.6 Hz, 1H), 4.58 (s, 2H), 3.61 – 3.46 (m, 4H), 2.70 (ddd, J = 13.1, 8.6, 6.3 Hz, 3H), 2.63 (dd, J = 11.2, 6.0 Hz, 4H), 2.23 (s, 3H), 1.63 (dt, J = 15.3, 7.5 Hz, 2H), 1.55 (d, J = 6.6 Hz, 6H), 1.09 (d, J = 6.5 Hz, 6H), 1.00 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.44, 165.96, 158.96, 154.78, 146.57, 143.29, 139.87, 136.47, 136.36, 132.35, 128.89, 125.52, 121.47, 120.52, 119.94, 109.10, 108.49, 106.74, 54.65, 50.45, 48.57 (two carbons), 45.59 (two carbons), 36.12, 35.28, 23.70, 22.20 (two carbons), 18.74, 18.62 (two carbons), 14.04. HPLC (method 1): 95%; t_R 4.45 min; MS (ESI): 570 $[M + H]^+$; HRMS calcd. for $C_{33}H_{43}N_7O_2$ + H: 570.3557; found: 570.3559 $[M + H]^+$.



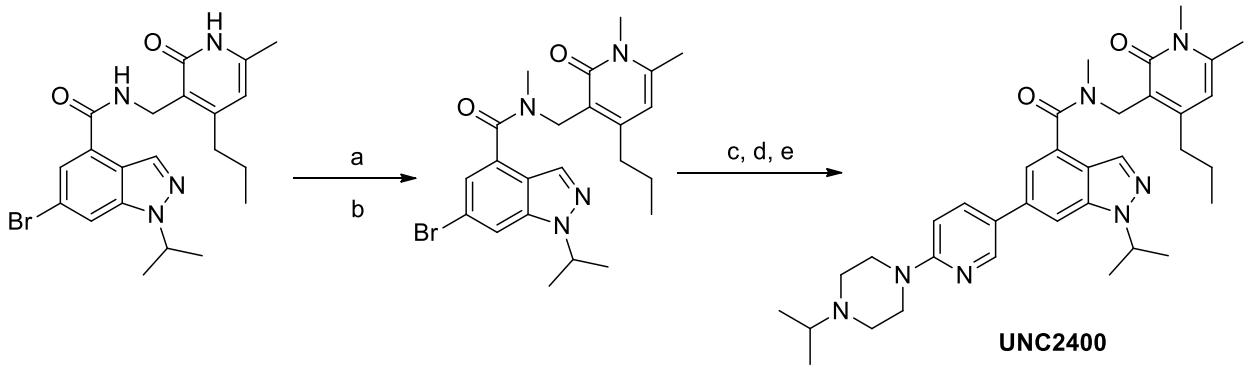
6-(5-Fluoropyridin-2-yl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (5). 6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (50 mg, 0.112 mmol), bis(pinacolato)diboron (34 mg, 0.135 mmol) and potassium acetate (16.5 mg, 0.168 mmol) were mixed with 1,4-dioxane (1.25 mL) under argon atmosphere at room temperature. The mixture was added [1,1-bis(diphenylphosphino) ferrocene]dichloropalladium(II), complex with dichloromethane (9.1 mg, 0.011 mmol) as catalyst. The resulting mixture was heated to 80°C. After being stirred overnight, the resulting mixture was concentrated *in vacuo*, which was used to the next reaction without further purification. To the solution of crude 1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indazole-4-carboxamide and 2-bromo-5-fluoropyridine (158 mg, 0.896 mmol) in 1,4-dioxane and water (1.25mL/0.25mL) was added tetrakis(triphenylphosphine)palladium (26 mg, 0.022 mmol) and potassium carbonate (46 mg, 0.336 mmol). After being stirred overnight at 90°C, the resulting mixture was purified by preparative HPLC (10% – 100% methanol / 0.1% TFA in H_2O) to afford compound **5** as a white solid (32 mg, 61% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.69 (s, 1H), 8.52 (d, J = 2.2 Hz, 1H), 8.44 (s, 1H), 8.22 (s, 1H), 8.07 (s, 1H), 7.94 (s, 1H), 7.85 (dd, J = 8.6, 4.1 Hz, 1H), 7.47 (td, J = 8.4, 2.3 Hz, 1H), 6.02 (s, 1H), 4.97 (dt, J = 13.2, 6.6 Hz, 1H), 4.69 (d, J = 3.8 Hz, 2H), 2.82 – 2.68 (m, 2H), 2.21 (s, 3H), 1.70 – 1.62 (m, 2H),

1.60 (d, J = 6.6 Hz, 6H), 1.02 (t, J = 7.3 Hz, 3H). HPLC (method 1): 95%; t_R 5.66 min; MS (ESI): 462 $[M + H]^+$.

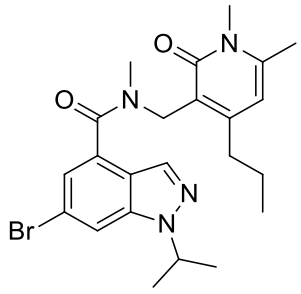


6-(4-Fluorophenyl)-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (6). To a microwave vessel containing 6-bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (50.6 mg, 0.112 mmol) was added K_2CO_3 (46.4 mg, 0.336 mmol), (4-fluorophenyl) boronic acid (23.5 mg, 0.168 mmol), and tetrakis(triphenylphosphine)palladium (1.16 mg, 0.001 mmol). After adding 1 mL of dioxane: H_2O (5:1), the vessel was placed in a microwave reactor for 20 minutes at 150°C. The content was separated using EtOAc and brine, extracting 3 times with EtOAc, then dried using Na_2SO_4. The crude product was purified using HPLC and then concentrated *in vacuo* to yield compound **6** as a white solid (37.7 mg, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (s, 1H), 7.88 (s, 2H), 7.72 (s, 1H), 7.61 (d, J = 9.5 Hz, 3H), 7.11 (s, 2H), 6.06 (s, 1H), 4.89 (dt, J = 13.3, 6.7 Hz, 1H), 4.68 (d, J = 5.4 Hz, 2H), 2.82 – 2.71 (m, 2H), 2.19 (s, 3H), 1.72 – 1.55 (m, 8H), 1.02 (t, J = 7.3 Hz, 3H). HPLC (method 1): 95%; t_R 5.86 min; MS (ESI): 461 $[M+H]^+$.

Scheme S2. Synthesis of UNC2400.

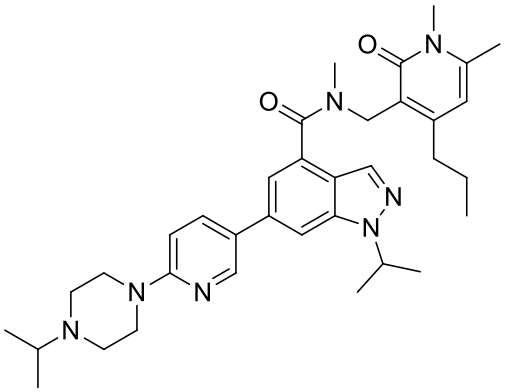


Reagents and conditions: (a) DMF, K_2CO_3, CH_3I, rt, 68%; (b) DMF, NaH, CH_3I, rt, 92%; (c) K_2CO_3, $Pd(PPh_3)_4$, 1-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine, 1,4-dioxane, H_2O, microwave, 150°C; (d) TFA, DCM, 88% (two steps); (e) acetone, MeOH, HOAc, $NaBH_3CN$, 0°C - rt, 83%.



6-Bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-N-methyl-1H-indazole-4-carboxamide. 6-Bromo-1-isopropyl-N-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1H-indazole-4-carboxamide (50.8 mg, 0.112 mmol), and K_2CO_3 (102.4 mg, 0.741 mmol) were added to a 4 mL scintillation vial and dissolved in DMF (0.5 mL). The resulting mixture was stirred at room temperature for 5 minutes before adding iodomethane (100 µL). The mixture was stirred at rt for 24 hours and purified by HPLC to yield 6-bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-1H-indazole-4-carboxamide (35.7 mg, 0.078 mmol, 68%). 6-Bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-1H-indazole-4-carboxamide (15 mg, 0.033 mmol) and

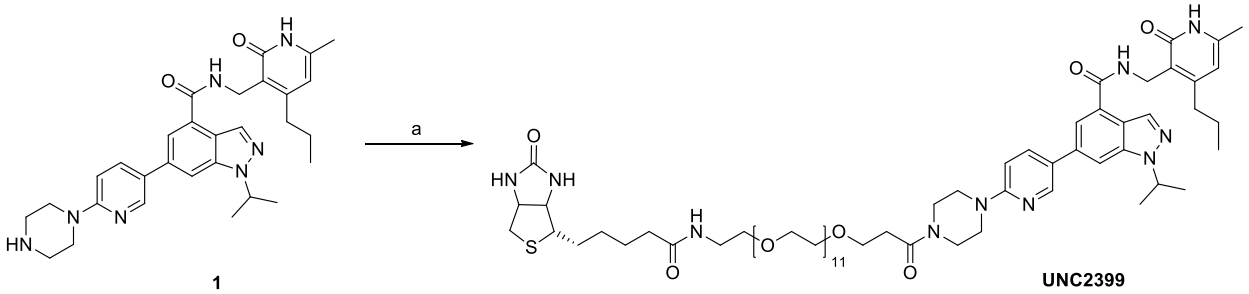
sodium hydride (2 mg, 0.050 mmol) were added to a 4 mL scintillation vial and were dissolved in DMF (0.15 mL). The mixture was stirred at rt for 5 minutes before adding iodomethane (10 μL). The resulting mixture was stirred at rt for 24 hours and purified by HPLC to yield the title compound as a yellow solid (14.4 mg, 0.030 mmol, 92%). ^{1}H NMR (the major rotamer is reported here) (400 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.64 (s, 1H), 7.25 (s, 1H), 6.12 (s, 1H), 4.85 (s, 2H), 4.81 – 4.66 (m, 1H), 3.59 (s, 3H), 2.93 (s, 3H), 2.70 (t, *J* = Hz, 2H), 2.38 (s, 3H), 1.76 – 1.46 (m, 8H), 1.01 (t, *J* = 7.3 Hz, 3H). HPLC (method 1): 95%; t_R 5.75 min; MS (ESI): 473 $[M+H]^+$.



N-((1,6-Dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-6-(6-(4-isopropylpiperazin-1-yl)pyridin-3-yl)-N-methyl-1H-indazole-4-carboxamide (UNC2400). 6-Bromo-N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-N-methyl-1H-indazole-4-carboxamide (22.6 mg, 0.034 mmol) was added to a microwave vessel with tert-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate (27.9 mg, 0.112 mmol), K_2CO_3 (23.2 mg, 0.168 mmol), and a catalytic amount of tetrakis(triphenylphosphine)palladium (1 mg, 0.001 mmol). The resulting mixture was dissolved in 1 mL of dioxane: H_2O (5:1) and placed in a microwave reactor at 150°C for 20 min. The resulting mixture was separated with EtOAc and brine, extracted 3 times with EtOAc and

purified by HPLC. The dried product was then dissolved in 1 mL TFA:EtOAc (1:1), basified using NH_3 (1 mL), and then concentrated *in vacuo* to yield N-((1,6-dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-N-methyl-6-(6-(piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (19.9 mg, 0.030 mmol, 88%). N-((1,6-Dimethyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1-isopropyl-N-methyl-6-(6-(piperazin-1-yl)pyridin-3-yl)-1H-indazole-4-carboxamide (19.9 mg, 0.030 mmol) was added to a vial containing sodium cyanoborohydride (15 mg, 0.239 mmol) on ice and dissolved in methanol (0.2 mL). Acetone (30 μL) and acetic acid (25 μL) were then added to the mixture, the vial was removed from ice and the reaction was run at rt overnight. The mixture was then purified using HPLC yielding UNC2400 as an off-white solid (14.9 mg, 0.025 mmol, 83%). ^{1}H NMR (the major rotamer is reported here) (400 MHz, $CDCl_3$) δ 8.47 (s, 1H), 7.93 (s, 1H), 7.76 (d, J = 8.7 Hz, 1H), 7.50 (s, 1H), 7.32 (s, 1H), 6.73 (d, J = 8.8 Hz, 1H), 6.00 (s, 1H), 4.98 – 4.79 (m, 3H), 3.63 (s, 3H), 3.55 (s, 3H), 2.94 (s, 3H), 2.83 – 2.60 (m, 7H), 2.35 (s, 3H), 1.83 – 1.52 (m, 9H), 1.10 (d, J = 6.5 Hz, 6H), 1.02 (t, J = 7.2 Hz, 3H). HPLC (method 2): 95%; t_R 4.36 min; MS (ESI): 598 $[M+H]^+$.

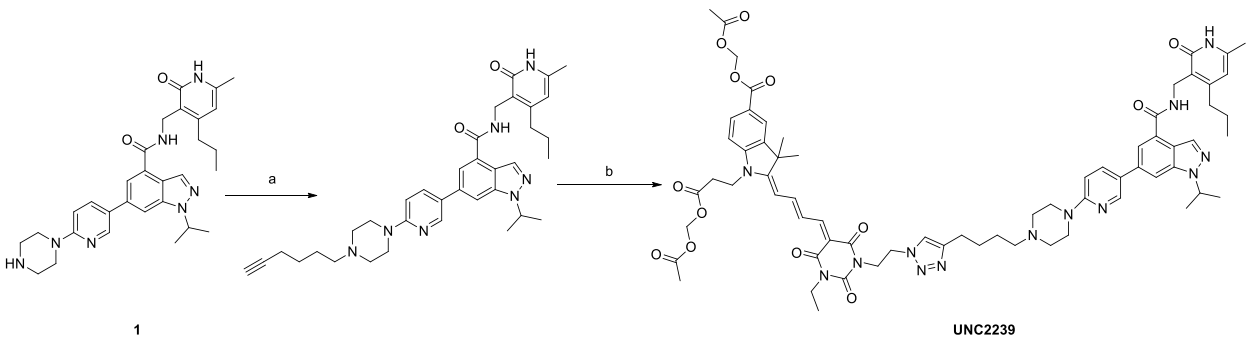
Scheme S3. Synthesis of UNC2399.



Reagents and conditions: (a) *O*-[2-(biotinylamino)ethyl]-*O*'-(2-carboxyethyl)undecaethylene glycol, DMF, DIPEA, HATU, rt, 41%.

UNC2399. Compound **1** (21.4 mg, 0.041 mmol) was added to a vial containing HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (18.5 mg, 0.049 mmol) and *O*-[2-(biotinylamino)ethyl]-*O*'-(2-carboxyethyl)undecaethylene glycol. The two components were dissolved in DMF (0.3 mL) and DIPEA (8.5 μL) was added to the resulting mixture. The reaction was run at room temperature for 24 hours, and purified by HPLC to yield UNC2399 as amber oil (22.6 mg, 0.016 mmol, 41%). ^{1}H NMR (400 MHz, d_4-MeOH) δ 8.51 (dd, J = 9.5, 2.4 Hz, 1H), 8.43 (d, J = 2.2 Hz, 1H), 8.40 (s, 1H), 8.10 (s, 1H), 7.80 (d, J = 1.3 Hz, 1H), 7.50 (d, J = 9.5 Hz, 1H), 6.18 (d, J = 0.7 Hz, 1H), 5.13 (tt, J = 13.4, 6.6 Hz, 2H), 4.61 (s, 2H), 4.49 (dd, J = 7.9, 4.2 Hz, 1H), 4.30 (dd, J = 7.9, 4.5 Hz, 1H), 3.97 – 3.78 (m, 9H), 3.67 – 3.55 (m, 33H), 3.52 (t, J = 5.5 Hz, 2H), 3.34 (t, J = 5.5 Hz, 2H), 3.19 (ddd, J = 8.9, 5.8, 4.6 Hz, 1H), 2.92 (dd, J = 12.8, 5.0 Hz, 1H), 2.79 – 2.67 (m, 5H), 2.28 (s, 3H), 2.20 (t, J = 7.4 Hz, 2H), 1.78 – 1.52 (m, 11H), 1.48 – 1.37 (m, 2H), 1.03 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, d_4-MeOH) δ 176.04, 173.00, 168.58, 166.00, 157.72, 153.54, 145.20, 143.90, 141.15, 136.66, 133.95, 133.83, 129.93, 127.56, 122.45, 120.26, 113.81, 111.28, 110.37, 71.55, 71.52, 71.50, 71.45, 71.35, 71.25, 70.56, 68.72, 63.36, 61.62, 57.00, 51.49, 47.04, 46.59, 45.73, 41.87, 41.07, 40.35, 36.71, 36.61, 36.10, 34.54, 29.76, 29.51, 26.84, 24.79, 22.54, 18.68, 14.40. HPLC (method 2): 95%; t_R 4.72 min; MS (ESI): 1354 $[M+H]^+$.

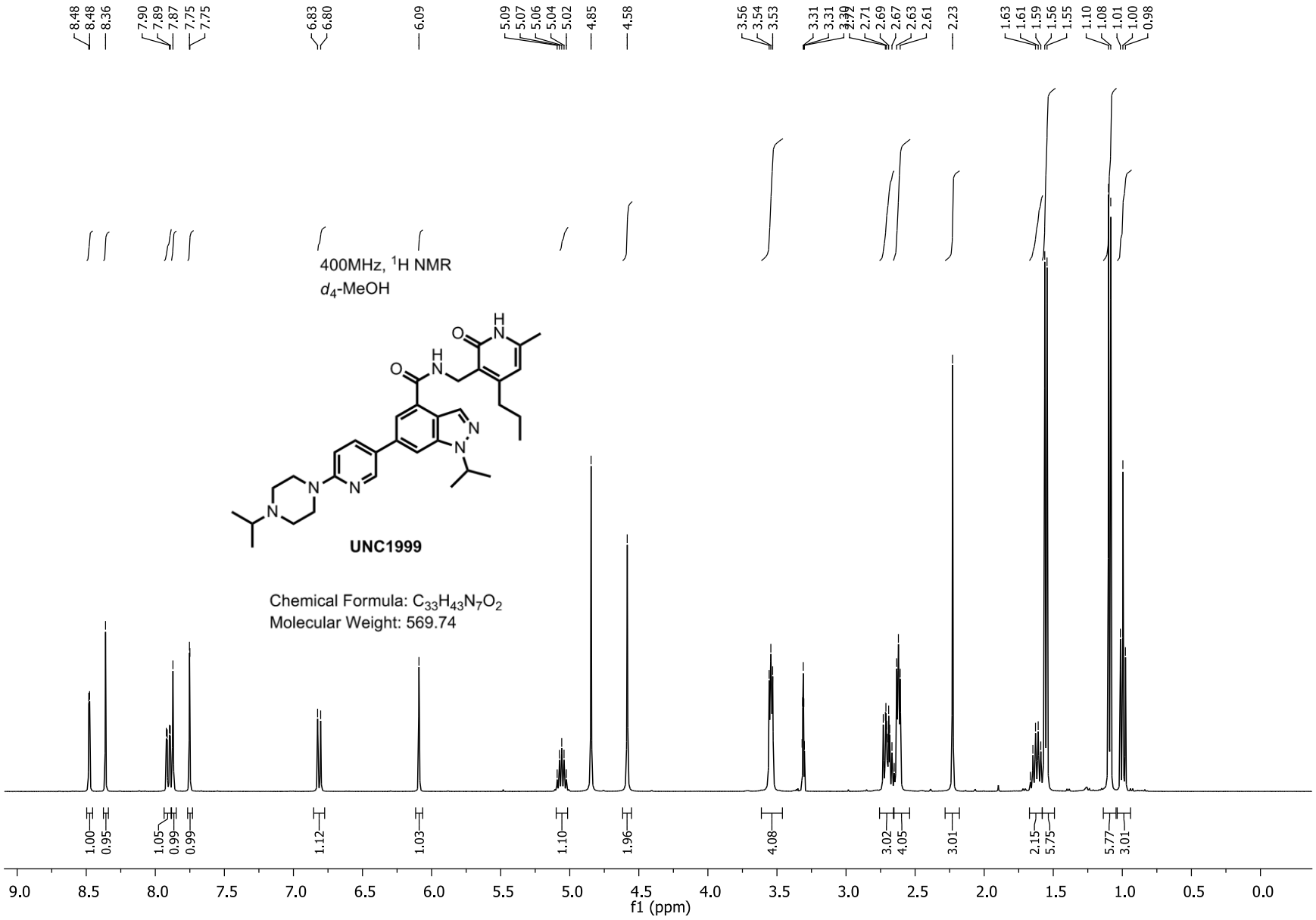
Scheme S4. Synthesis of UNC2239.



Reagents and conditions: (a) hex-5-yn-1-yl 4-bromobenzenesulfonate, K_2CO_3, DMF, rt, 74%; (b) mero166, *cat*. $Cu(CH_3CN)_4PF_6$, DCM, rt, 90%.

6-(6-(4-(Hex-5-yn-1-yl)piperazin-1-yl)pyridin-3-yl)-1-isopropyl-*N*-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1*H*-indazole-4-carboxamide. To a solution of hex-5-yn-1-ol (2.07 g, 21.09 mmol) in dichloromethane (30 mL) was added 4-bromobenzenesulfonyl chloride (5.93g, 23.20 mmol) and DABCO (2.84 g, 25.30 mmol) at 0ºC. The resulting mixture was stirred for 2 hours at 0ºC. 15 mL of distilled water was added. The organic phase was dried with sodium sulfate, filtered, concentrated and purified by silica gel chromatography (hexanes to 15% ethyl acetate in hexanes) to afford hex-5-yn-1-yl 4-bromobenzenesulfonate (6.15 g, 92%). To a solution of compound **1** (56 mg, 0.10 mmol) and hex-5-yn-1-yl 4-bromobenzenesulfonate (44 mg, 0.14 mmol) in 1.0 mL of DMF was added K_2CO_3 (41 mg, 0.30 mmol) at rt. The resulting mixture was stirred overnight at rt. After water (2 mL) was added, the mixture was extracted with ethyl acetate (10 mL × 3). The combined organic phases were dried with sodium sulfate, filtered, concentrated and purified by preparative HPLC to give the title compound as a yellow solid (44 mg, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.93 (s, 1H), 8.47 (dd, *J* = 2.5, 0.4 Hz, 1H), 8.39 (s, 1H), 8.01 (t, *J* = 5.7 Hz, 1H), 7.75 (dd, *J* = 8.8, 2.6 Hz, 1H), 7.71 (d, *J* = 1.3 Hz, 1H), 7.57 (s, 1H), 6.66 (d, *J* = 8.8 Hz, 1H), 5.92 (d, *J* = 0.6 Hz, 1H), 4.85 (dd, *J* = 13.3, 6.7 Hz, 1H), 4.66 (d, *J* = 5.7 Hz, 2H), 3.65 – 3.56 (m, 4H), 2.70 (dd, *J* = 8.5, 7.0 Hz, 2H), 2.63 – 2.51 (m, 4H), 2.48 – 2.38 (m, 2H), 2.23 (td, *J* = 6.9, 2.6 Hz, 2H), 2.15 (s, 3H), 1.96 (t, *J* = 2.6 Hz, 1H), 1.73 – 1.52 (m, 12H), 1.00 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.37, 166.01, 158.89, 154.68, 146.65, 143.25, 139.91, 136.57, 136.35, 132.32, 129.01, 125.81, 121.59, 120.54, 120.06, 109.11, 108.58, 106.81, 84.34, 68.68, 58.14, 53.01, 50.49, 45.17, 36.25, 35.33, 26.47, 25.81, 23.76, 22.25, 18.81, 18.44, 14.07. HPLC (method 1): 96%; t_R 4.79 min; MS (ESI): 608 $[M+H]^+$.

UNC2239. $Cu(CH_3CN)_4PF_6$ (0.7 mg, 0.0018 mmol) was added to a solution of compound 6-(6-(4-(hex-5-yn-1-yl)piperazin-1-yl)pyridin-3-yl)-1-isopropyl-*N*-((6-methyl-2-oxo-4-propyl-1,2-dihydropyridin-3-yl)methyl)-1*H*-indazole-4-carboxamide (6.3 mg, 0.0104 mmol) and mero166 (5.9 mg, 0.0087 mmol) in DCM (1.0 mL). The resulting mixture was stirred overnight at rt, concentrated and purified by preparative HPLC to give UNC2239 as a dark red solid (10.1 mg, 90%). ^{1}H NMR (400 MHz, d_4-MeOH) δ 8.55 (s, 1H), 8.33 (s, 1H), 8.11–8.04 (m, 1H), 8.04–7.93 (m, 3H), 7.91 (s, 1H), 7.83 (s, 1H), 7.80–7.63 (m, 3H), 7.24–7.17 (m, 1H), 7.06 (d, J = 8.9 Hz, 1H), 6.23–6.07 (m, 2H), 5.95 (d, J = 3.5 Hz, 2H), 5.68 (d, J = 8.6 Hz, 2H), 5.13–5.02 (m, 1H), 4.71–4.63 (m, 2H), 4.62 (s, 2H), 4.43–4.21 (m, 5H), 3.88–3.77 (m, 2H), 3.75–3.32 (m, 8H), 3.27–3.18 (m, 2H), 2.86 (dd, J = 14.5, 7.0 Hz, 2H), 2.82–2.73 (m, 3H), 2.27 (s, 3H), 2.11 (s, 3H), 2.00 (d, J = 3.8 Hz, 3H), 1.89–1.71 (m, 4H), 1.74–1.61 (m, 8H), 1.58 (d, J = 6.6 Hz, 6H), 1.11 (t, J = 7.0 Hz, 3H), 1.03 (t, J = 7.3 Hz, 3H). MS (ESI): 1289 $[M+H]^+$.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7.87
7.75
7.75
6.83
6.80
6.09
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5.06
5.04
5.02
4.85
4.58
3.56
3.54
3.53
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3.31
2.71
2.69
2.67
2.63
2.61
2.23
1.63
1.61
1.59
1.56
1.55
1.10
1.08
1.01
1.00
0.98
400MHz, ^{1}H NMR
d_4-MeOH
UNC1999
Chemical Formula: $C_{33}H_{43}N_7O_2$
Molecular Weight: 569.74
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1.05
0.99
0.99
1.12
1.03
1.10
1.96
4.08
3.02
4.05
3.01
2.15
5.75
5.77
3.01
9.0
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6.5
6.0
5.5
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2.5
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1.0
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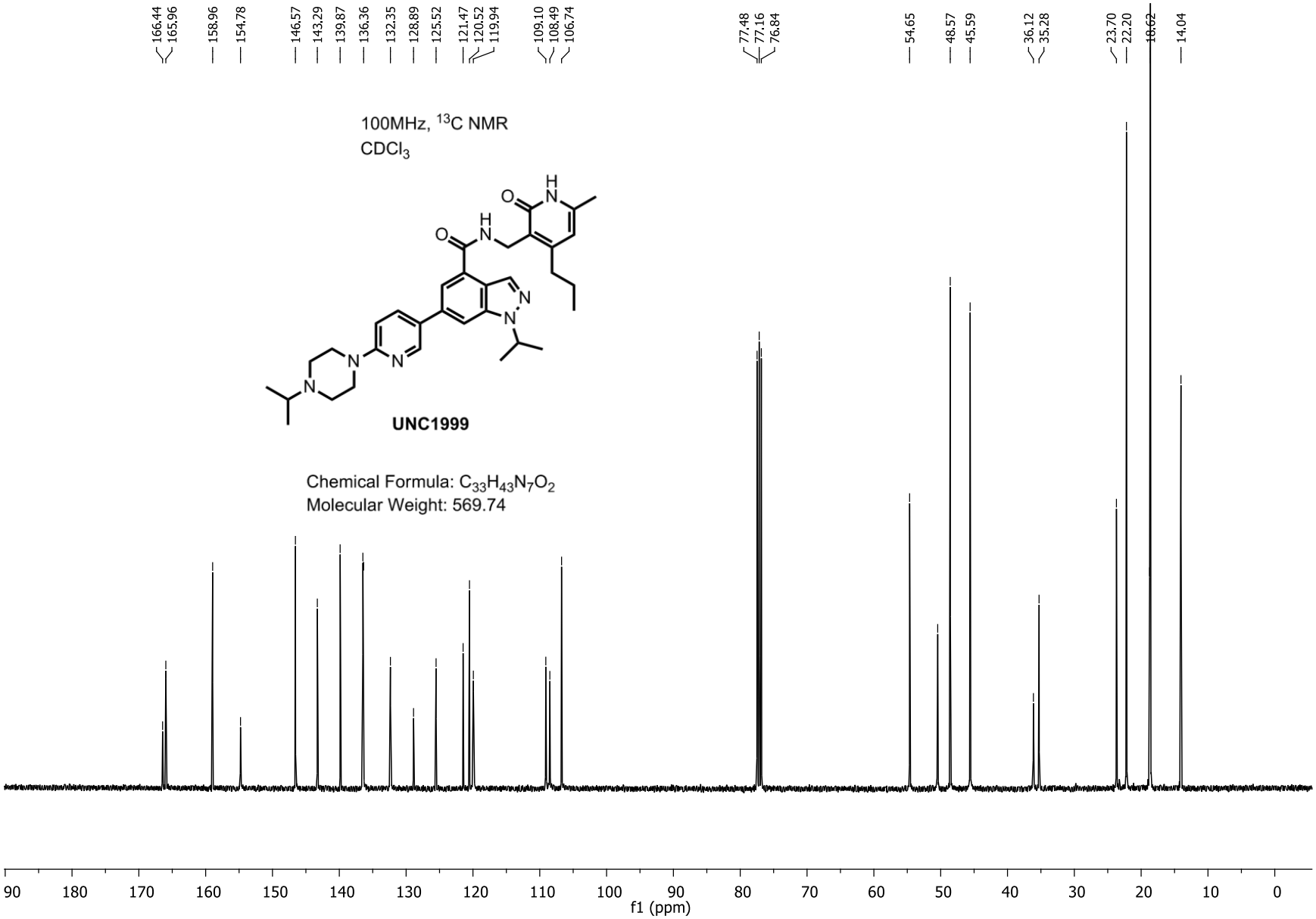
0.0
f1 (ppm)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109.10
108.49
106.74
77.48
77.16
76.84
54.65
48.57
45.59
36.12
35.28
23.70
22.20
18.62
14.04
100MHz, 13C NMR
CDCl3
UNC1999
Chemical Formula: C33H43N7O2
Molecular Weight: 569.74
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160
150
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110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

EZH2 Homology Modeling

An EZH2 homology model was built using the X-ray crystal structure of the lysine methyltransferase GLP (PDB: 2RFI) as a template. 3D conformation of EPZ005687 or UNC1999 was constructed and docked into the EZH2 homology model. A subsequent induced-fit minimization was conducted to further optimize the complex. Ligand interaction diagram was drawn with Maestro 9.3 (Schrodinger LLC, www.schrodinger.com).

Scintillation Proximity Assay

Methyltransferase activity assays were performed by monitoring the incorporation of tritium-labeled methyl group from *S*-adenosylmethionine (^{3}H-SAM) to biotinylated peptide substrates using Scintillation Proximity Assay (SPA) for PRC2-EZH2 trimeric complex (EZH2:EED:SUZ12), PRC2-EZH1 pentameric complex (EZH1:EED:SUZ12:RBBP4:AEBP2), SETD7, G9a, GLP, SETDB1, SETD8, SUV420H1, SUV420H2, SUV39H2, MLL1 tetrameric complex (MLL:WDR5:RbBP5:ASH2L), PRMT1, PRMT3, PRMT5-MEP50 complex and SMYD2. The reaction buffer for SMYD2 and SMYD3 was 50 mM Tris pH 9.0, 5 mM DTT, 0.01%TritonX-100; for G9a, GLP and SUV39H2 was 25 mM potassium phosphate pH 8.0, 1 mM EDTA, 2 mM MgCl2 and 0.01% Triton X-100; and for other HMTs 20 mM Tris pH 8.0, 5 mM DTT, 0.01% TritonX-100. To stop the enzymatic reactions, 10 μL of 7.5 M guanidine hydrochloride was added, followed by 180 μL of buffer, mixed and transferred to a 384-well FlashPlate (Cat.# SMP103; Perkin Elmer; www.perkinelmer.com). After mixing, the reaction mixtures were incubated and the CPM counts were measured using Topcount plate reader (Perkin Elmer, www.perkinelmer.com). The CPM counts in the absence of compound for each data set were defined as 100% activity. In the absence of the enzyme, the CPM counts in each data set were defined as background (0%). IC_{50} values were determined using compound

concentrations ranging from 100 nM to 100 μM. The IC_{50} values were determined using SigmaPlot software. EZH2-Y641F assays were performed using 30 nM of enzyme in 20 mM Tris pH 8, 5 mM DTT, 0.01% Triton X-100, 5 μM SAM and 1 μM of H3 (1-24) peptide (same as for the wild-type PRC2-EZH2 complex). For DNMT1, the assay was performed as described above using hemimethylated dsDNA as a substrate. The dsDNA substrate was prepared by annealing two complementary strands (biotintlated forward strand: B-GAGCCCGTAAGCCCGTTCAGGTCG and reverse strand: CGACCTGAACGGGCTTACGGGCTC), synthesized by Eurofins MWG Operon. Reaction buffer was 20 mM Tris-HCl, pH 8.0, 5mM DTT, 0.01% Triton X-100. Methyltransferase activity assays for DOT1L was performed using Filter-plates (Millipore; cat.# MSFBN6B10; www.millipore.com). Reaction mixtures in 20 mM Tris-HCl, pH 8.0, 5 mM DTT, 2 mM $MgCl_2$ and 0.01% Triton X-100 were incubated at room temperature for 1h, 100 μL 10% TCA was added, mixed and transferred to filter-plate. Plates were centrifuged at 2000 rpm for 2 min followed by 2 additional 10% TCA wash and one ethanol wash (180 μL) followed by centrifugation. Plates were dried and 100 μL MicroO was added and centrifuged. 70 μL MicroO was added and CPM were measured using Topcount plate reader.

Mechanism of Action Studies

To determine the mechanism of action of UNC1999, methyltransferase activity of PRC2 – EZH2 was assessed in the presence of different concentrations of the compound (0, 1, 3 and 5 nM) at (1) fixed concentration of peptide (5 μM) and SAM concentrations from 0.625 to 10 μM and (2) fixed concentration of SAM (10 μM) and peptide concentrations from 0.3 to 5 μM. Assays were performed in triplicate, and kinetic values were obtained using Lineweaver-Burk plots (SigmaPlot, Enzyme Kinetics Module). Methyltransferase activity of PRC2 - EZH1 was assessed

in the presence of different concentrations of UNC1999 (0, 10, 20, 60, and 80 nM) at (1) fixed concentration of peptide (5 μM) and SAM concentrations from 0.5 to 20 μM and (2) fixed concentration of SAM (20 μM) and peptide concentrations from 0.3 to 10 μM. Assays were performed in triplicate.

EZH2 Y641N Biochemical Assay

EZH2 Y641N mutant was generated and assayed by BPS Bioscience. A series of dilutions of UNC1999 were prepared with 10% DMSO in HMT assay buffer (BPS #52170) and 5 μL of the dilution was added to a 50 μL reaction so that the final concentration of DMSO is 1% in all of reactions. All of the enzymatic reactions were conducted in duplicate at room temperature for 60 minutes (EZH2; BPS #51004) and 180 minutes (EZH2 Y641N; BPS #51028) in a 50 μL mixture containing HMT assay buffer, S-adenoslymethionine (BPS #52120), enzyme, and UNC1999. These 50 μL reactions were carried out in wells of a HMT substrate pre-coated plate. After enzymatic reactions, the reaction mixtures were discarded and each of the wells was washed three times with TBST buffer, and slowly shaken with Blocking Buffer (BPS #52100) for 10 minutes. Wells were emptied, and 100 μL of diluted 1° antibody (BPS #52140F) was added. The plate was then slowly shaken for 60 minutes at room temperature. As before, the plate was emptied and washed three times, and shaken with Blocking Buffer for 10 minutes at room temperature. After discarding the Blocking Buffer, 100 μL of diluted 2° antibody (BPS #52131H) was added. The plate was then slowly shaken for 30 minutes at room temperature. As before, the plate was emptied and washed three times, and shaken with Blocking Buffer for 10 minutes at room temperature. Blocking Buffer was discarded and a mixture of the HRP chemiluminescent substrates was freshly prepared. 100 μL of this mixture was added to each empty well.

Immediately, the luminescence of the samples was measured in a BioTek Synergy™ 2 microplate reader.

Kinase Selectivity Assays. Selectivity of UNC1999 against a panel of 50 kinases was conducted by Carna Biosciences, Inc. using a standard off-chip mobility shift assay technology. The full list of the 50 kinases is included in Supporting Table S1.

NIMH PDSP Selectivity Assays. Selectivity of UNC1999 against the 44 targets in the NIMH PDSP selectivity panel was performed by the NIMH PDSP at University of North Carolina at Chapel Hill (http://pdsp.med.unc.edu/) in radioligand binding assays. The full list of the 44 targets is included in Supporting Table S2. Protocols of the binding and functional assays are available from the NIMH PDSP website: http://pdsp.med.unc.edu/UNC-CH%20Protocol%20Book.pdf.

Pharmacokinetic Studies

Standard PK studies were conducted using male Swiss albino mice at Sai Life Sciences. Four doses (15, 50, and 150 mg/kg IP, and 50 mg/kg PO) of UNC1999 were evaluated. Each study lasted 24 h. Plasma concentrations of UNC1999 reported at each of the eight time points (0.08, 0.25, 0.5, 1, 2, 4, 8, and 24 h post dosing) are the average values from 3 test animals.

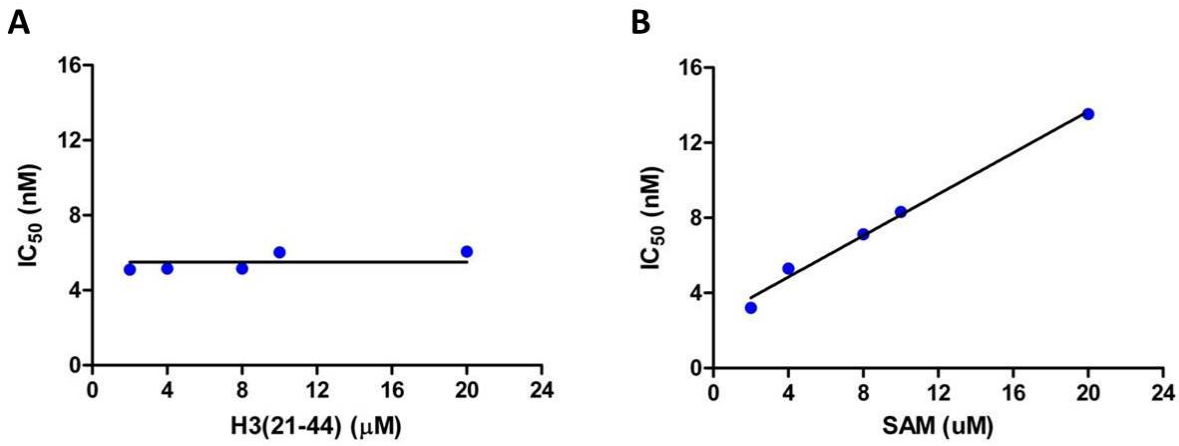
Cell Culture and Treatment with Imaging Probes (UNC2239 Studies)

Human embryonic kidney (HEK) 293 cells and NIH 3T3 mouse embryonic fibroblasts (MEF) stably expressing YPet fluorophore were maintained in 10% CO_2 at 37 ^{o}C in Dulbecco's modified Eagle's medium (DMEM, Cellgro) with 10% fetal bovine serum (HyClone, Thermo Scientific) and 2 mM GlutaMax (Gibco, Life Technologies). HEK 293 cells were transfected with GFP-tagged EZH2 in the pCMV6-AC-GFP vector (OriGene, cat. # RG202054) using X-TremeGENE 9 (Roche) at a final concentration of 1 μg DNA/well of a 6-well cell culture plate.

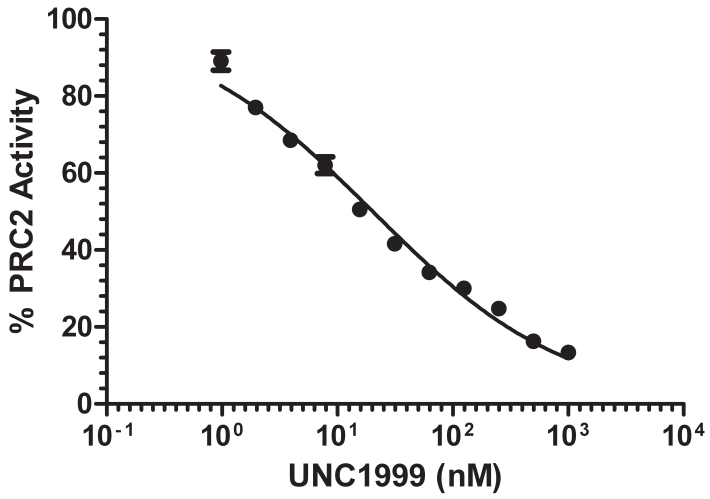
MEF cells were plated 3-4 hours before imaging on coverslips coated with fibronectin 20 μg/mL (Sigma-Aldrich) overnight. The cells were incubated for 15 min with 3.75 μM EZH2 probe (or 1 μM for Hoechst) in 10% CO_2 at 37 ^oC in imaging medium [HAM'S F-12 (K) Medium (SKU #RR080058L1, Gibco, Life Technologies) with 5% fetal bovine serum (HyClone, Thermo Scientific) and 2 mM GlutaMax (Gibco, Life Technologies)]. The cells were then gently washed with imaging medium (2 X 2 mL) and imaged in the same medium.

Image Acquisition

Live cell imaging was carried out on an Olympus IX81 microscope with UPLFLN 40X oil objective (NA 1.3) and mercury lamp excitation (103W HBO bulb). Filters used for the EZH2-dye probe (UNC2239) and the dye alone were 545/50 excitation and 620/60 emission. Excitation was through a ND2.0 (1% transmission) neutral density filter, with 150 ms exposure (for EZH2-dye probe acquisition) or 50 ms (for dye alone acquisition). For YPet, filters were 500/20 excitation and 535/30 emission. Excitation was through a ND2.0 (1%T) filter with 500 ms exposure (when acquiring EZH2-dye probe) or 150 ms (when acquiring dye alone). For EZH2-GFP probe imaging, the filters used were 470/40 excitation and 525/50 emission. Excitation was through a ND1.3 (5%T) neutral density filter with 500 ms exposure. Imaging of Hoechst was carried out using 360-370 excitation, 420-460 emission, ND2.0 (1%T), and 100 ms exposure. Images were acquired with a Coolsnap ES camera (Photometrics) with a Sony 6.45 x 6.45 μM/pixel chip using 2 x 2 binning. All image acquisition, processing, and analysis was carried out with Metamorph software. Ratio images were generated from unprocessed images following shade correction, background subtraction, masking, registration for image alignment, and photobleach correction. The display threshold of ratio images was restricted to the pixels falling between 5 - 95% of the overall frequency histogram.

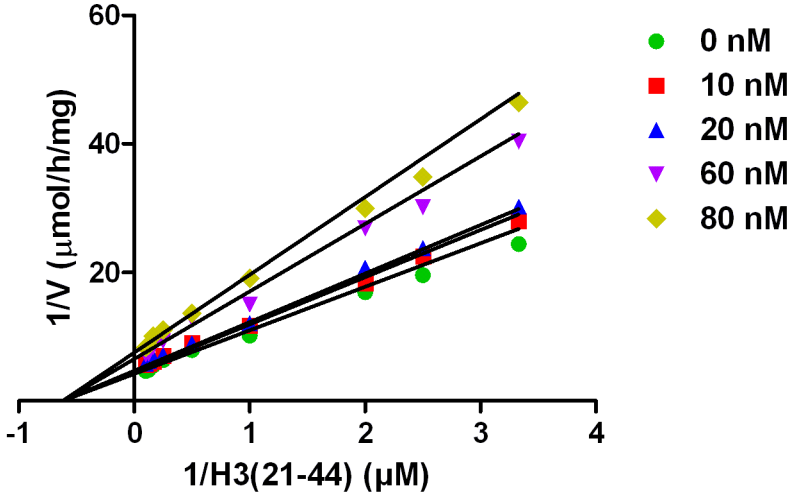


Supporting Figure S1. (A) Increasing the H3 peptide concentration had no effect on the IC_{50} values of UNC1999. (B) Increasing the SAM concentration had a positive linear relationship with the IC_{50} values of UNC1999.

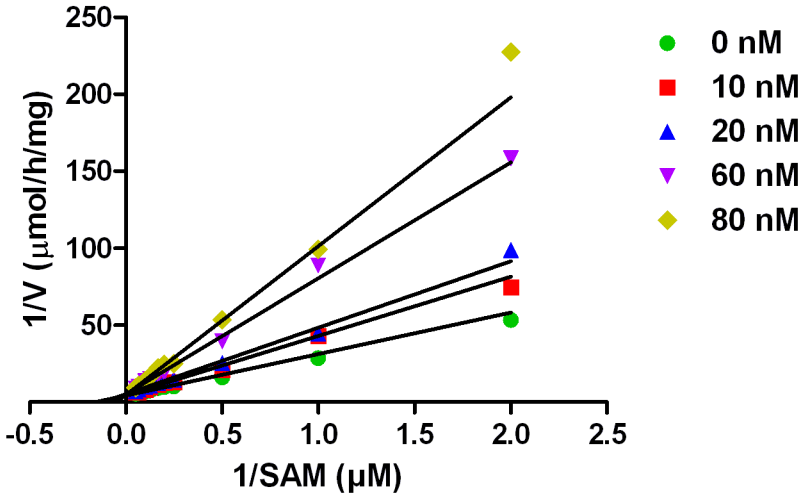


Supporting Figure S2. UNC1999 had high in vitro potency (IC_{50} < 15 nM) for the EZH2 Y641F mutant enzyme.

A

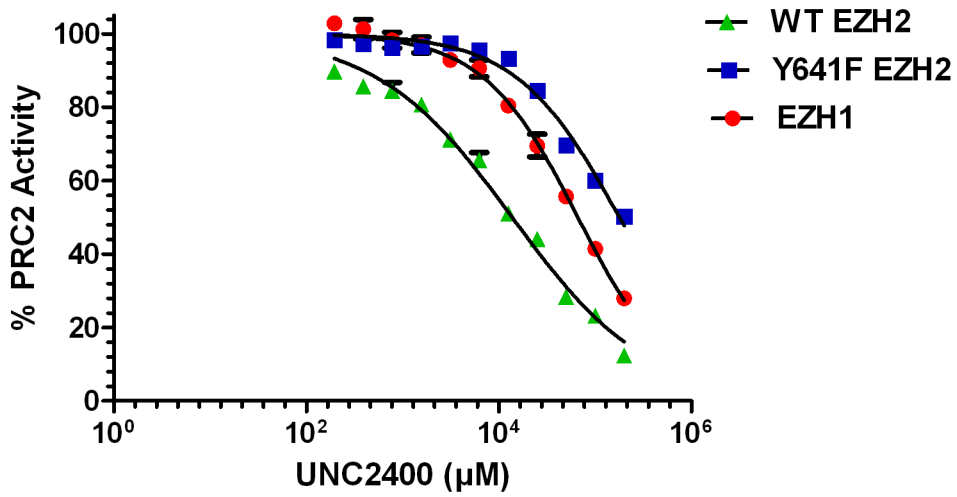


B

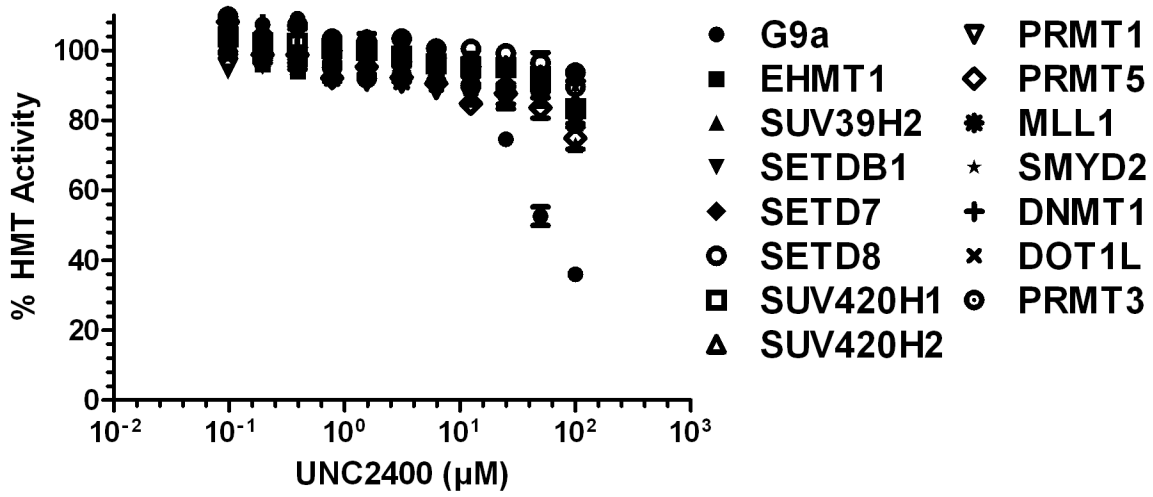


Supporting Figure S3. MOA of EZH1 inhibition by UNC1999. UNC1999 had high in vitro potency (IC_{50} < 15 nM) for the EZH2 Y641F mutant enzyme. Lineweaver-Burk plots demonstrated that UNC1999 is non-competitive with the histone H3 substrate (A), and competitive with the co-factor SAM (B).

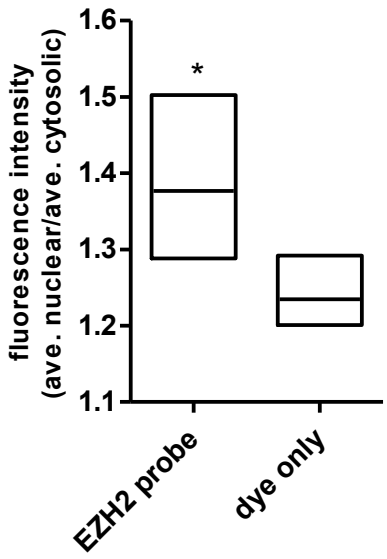
A



B



Supporting Figure S4. Selectivity of UNC2400. (A) UNC2400 had poor potencies for EZH2 Y641 and EZH1. (G) UNC2400 had negligible activities for 15 other methyltransferases.



Supporting Figure S5. Quantification of the average fluorescence intensity per unit area within the nuclear region versus the cytosolic region in YPet-MEF cells ($p < 0.05$, $n = 6$). Floating bars range from minimum to maximum values with the central transecting line at the mean value.

Supporting Table S1. Kinase Selectivity of UNC1999.

Kinase	10 μM UNC1999 (% inhibition)	Kinase	10 μM UNC1999 (% inhibition)
ABL	-4	**CHK1**	-2
CSK	-4	**CK1ε**	-4
EGFR	-3	**DAPK1**	-4
EPHA2	-6	**DYRK1B**	-4
EPHB4	-6	**Erk2**	3
FGFR1	-9	**GSK3β**	-13
FLT3	16	**HGK**	-2
IGF1R	-7	**IKKβ**	-3
ITK	-2	**IRAK4**	-7
JAK3	-8	**JNK2**	5
KDR	-2	**MAPKAPK2**	4
LCK	0	**MST1**	-5
MET	-3	**NEK2**	-4
PDGFRα	-5	**p38α**	0
PYK2	-5	**p70S6K**	6
SRC	-1	**PAK2**	4
SYK	-6	**PBK**	-10
TIE2	-6	**PDK1**	-6
TRKA	7	**PIM1**	1
TYRO3	-1	**PKACα**	-12
AKT1	-6	**PKCα**	-4
AMPKα1/β1/γ1	-3	**PKD2**	5
AurA	-4	**ROCK1**	5
CaMK4	20	**SGK**	-3
CDK2/CycA2	-3	**TSSK1**	5

Supporting Table S2. Selectivity of UNC1999 versus the NIMH-PDSP panel.

	5-HT1A	**5-HT1B**	**5-HT1D**	**5-HT1E**	**5-HT2A**	**5-HT2B**	**5-HT2C**	**5-HT3**	**5-HT5A**	**5-HT6**	**5-HT7**
	11	44	50	2	39	-9	22	12	10	11	-4
10 μM	**Alpha1A**	**Alpha1B**	**Alpha1D**	**Alpha2A**	**Alpha2B**	**Alpha2C**	**Beta1**	**Beta2**	**Beta3**	**BZP**	**D1**
UNC1999	3	6	14	14	48	47	-15	14	10	-6	13
(% inhibition)	**D2**	**D3**	**D4**	**D5**	**DAT**	**DOR**	**GABAA**	**H1**	**H2**	**H3**	**KOR**
	9	28	2	20	36	21	-17	16	30	95	34
	M1	**M2**	**M3**	**M4**	**M5**	**MOR**	**NET**	**PBR**	**SERT**	**Sigma1**	**Sigma2**
	18	41	10	21	9	13	83	31	6	57	93

Supporting Table S3. Binding affinities of UNC1999 versus H_3, NET, sigma1, and sigma2 and activity of UNC1999 in H_3 functional assays.

Target	Binding Affinity (K_i, nM)	Functional Activity
H_3	300	No agonist or antagonist activity at concentrations up to 1 μM
NET	1500	NT
Sigma1	4700	NT
Sigma2	65	NA

NT = not tested

NA = not available

References

1. Verma, S. K., Tian, X., LaFrance, L. V., Duquenne, C., Suarez, D. P., Newlander, K. A., Romeril, S. P., Burgess, J. L., Grant, S. W., Brackley, J. A., Graves, A. P., Scherzer, D. A., Shu, A., Thompson, C., Ott, H. M., Aller, G. S. V., Machutta, C. A., Diaz, E., Jiang, Y., Johnson, N. W., Knight, S. D., Kruger, R. G., McCabe, M. T., Dhanak, D., Tummino, P. J., Creasy, C. L., and Miller, W. H. (2012) Identification of Potent, Selective, Cell-Active Inhibitors of the Histone Lysine Methyltransferase EZH2, *ACS Med Chem Lett 3*, 1091-1096.